

# $\gamma$ -Aminobutyric acid B receptor 1 mediates behavior-impairing actions of alcohol in *Drosophila*: Adult RNA interference and pharmacological evidence

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In addition to their physiological function, metabotropic receptors for neurotransmitter  $\gamma$ -aminobutyric acid (GABA), the GABA<sub>B</sub> receptors, may play a role in the behavioral actions of addictive compounds. Recently, GABA<sub>B</sub> receptors were cloned in fruit flies (*Drosophila melanogaster*), indicating that the advantages of this experimental model could be applied to GABA<sub>B</sub> receptor research. RNA interference (RNAi) is an endogenous process triggered by double-stranded RNA and is being used as a tool for functional gene silencing and functional genomics. Here we show how cell-nonautonomous RNAi can be induced in adult fruit flies to silence a subtype of GABA<sub>B</sub> receptors, GABA<sub>B</sub>R1, and how RNAi combined with pharmacobehavioral techniques (including intra-abdominal injections of active compounds and a computer-assisted quantification of behavior) can be used to functionally characterize these receptors. We observed that injection of double-stranded RNA complementary to GABA<sub>B</sub>R1 into adult *Drosophila* selectively destroys GABA<sub>B</sub>R1 mRNA and attenuates the behavioral actions of the GABA<sub>B</sub> agonist, 3-aminopropyl-(methyl)phosphinic acid. Moreover, both GABA<sub>B</sub>R1 RNAi and the GABA<sub>B</sub> antagonist CGP 54626 reduced the behavior-impairing effects of ethanol, suggesting a putative role for the *Drosophila* GABA<sub>B</sub> receptors in alcohol's mechanism of action. The *Drosophila* model we have developed can be used for further *in vivo* functional characterization of GABA<sub>B</sub> receptor subunits and their involvement in the molecular and systemic actions of addictive substances.

fruit fly | GABA<sub>B</sub> receptors | ethanol | CGP 54626 | 3-APMPA

Fruit flies are used for research primarily because of the homology of *Drosophila* genes to those of mammals and because *Drosophila* is amenable to genetic manipulation including gene silencing through a process known as RNA interference (RNAi) (1–7). Typically, RNAi is triggered by double-stranded RNA (dsRNA), which is first processed by an RNase, Dicer (8), into 21- to 23-nt fragments. These fragments form a silencing complex that binds specifically to the dsRNA-complementary endogenous mRNA and leads to the destruction of the mRNA (1, 9). Injecting adult *Drosophila* intraabdominally with dsRNA results in the cell-nonautonomous silencing of the complementary endogenous mRNA throughout the body, including the CNS (10). Because this method does not interfere in the normal development of the animal but can replicate typical phenotypes produced by gene mutations (11), here we have used adult RNAi to silence  $\gamma$ -aminobutyric acid (GABA) B receptors.

The slow inhibitory GABA synaptic neurotransmission is mediated by the metabotropic G protein-coupled and cAMP-linked GABA<sub>B</sub> receptors (12, 13) that have been cloned in humans (14) and also recently in *Drosophila* (15). In addition to their physiological function, the GABA<sub>B</sub> receptors may play a role in the behavioral actions of addictive compounds such as ethanol (16–19). Although *Drosophila* has been used to study the mechanisms of action of alcohol (20–23), as yet, this model has not been used to characterize the involvement of GABA<sub>B</sub> receptors.

Mezler *et al.* (15) reported the cloning of three *Drosophila* GABA<sub>B</sub> receptor subunits: D-GABA<sub>B</sub>R1, D-GABA<sub>B</sub>R2, and D-GABA<sub>B</sub>R3. D-GABA<sub>B</sub>R1 and D-GABA<sub>B</sub>R2 show high sequence homology to mammalian GABA<sub>B</sub>R1 and GABA<sub>B</sub>R2, respectively; the subunit D-GABA<sub>B</sub>R3 seems to be an insect-specific subtype with no known mammalian counterpart and no known function. All three D-GABA<sub>B</sub> subunits are expressed in the CNS; *in situ* hybridization of *Drosophila* embryos showed that D-GABA<sub>B</sub>R1 and D-GABA<sub>B</sub>R2 are expressed in similar regions, whereas the D-GABA<sub>B</sub>R3 displayed a unique expression pattern. Moreover, these receptors were functional only when D-GABA<sub>B</sub>R1 and D-GABA<sub>B</sub>R2 were coexpressed either in *Xenopus laevis* oocytes or mammalian cell lines, whereas D-GABA<sub>B</sub>R3 was inactive in any combination. The *in vitro* pharmacology of the coexpressed D-GABA<sub>B</sub>R1/R2 subunits was slightly different from the pharmacology of the mammalian GABA<sub>B</sub> receptors; e.g., baclofen was not effective as an agonist. However, similar to mammalian receptors, the *Drosophila* receptors were also activated by 3-aminopropyl-(methyl)phosphinic acid (3-APMPA) and inhibited by CGP 54626 (15).

The mammalian GABA<sub>B</sub>R1 is responsible for GABA and ligand binding and mice lacking this subtype do not respond to typical GABA<sub>B</sub> agonists. Moreover, in the absence of drug treatment these mice also express numerous pathologic behaviors such as epilepsy, hyperalgesia, and impaired memory (24, 25). To circumvent these problems, which could be due to compensatory developmental responses of knockout mice, we planned our experiments such that RNAi was used for silencing of GABA<sub>B</sub>R1 subunits only in adult *Drosophila*.

## Materials and Methods

***Drosophila* and Injections.** Canton-S flies were cultured at 25°C and 50–60% humidity, on a 12 h light/12 h dark cycle, and on yeast, dark corn syrup, and agar food. Studies were performed with 5- to 7-day-old males. For injections, flies were anesthetized by CO<sub>2</sub> (maximally for 5 min). Using custom-beveled glass pipettes (20 × 40  $\mu$ m tip diameter) coupled to a cell injector and a micromanipulator, we injected a volume of 0.2  $\mu$ l per fly by a pulse pressure of 300 kPa under a stereo microscope (10). Drugs were prepared as 10× stock solutions; 0.2  $\mu$ l was injected per fly (the estimated volume of a fly is 2  $\mu$ l). Then, 3-APMPA (also known as SKF-97541; Sigma), CGP 54626 (Tocris Cookson, Ellisville, MO), and ethanol (Sigma) were dissolved/diluted with Ringer's solution (7.5 g/liter NaCl/0.35 g/liter KCl/0.21 g/liter CaCl<sub>2</sub>; pH 7.6–7.8; sterile-filtered). The stock solutions of drugs were prepared in DMSO; DMSO concentration in final solutions

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Abbreviations: GABA,  $\gamma$ -aminobutyric acid; 3-APMPA, 3-aminopropyl-(methyl)phosphinic acid; dsRNA, double-stranded RNA; GABA<sub>B</sub>R, GABA<sub>B</sub> receptor; D-GABA<sub>B</sub>R, *Drosophila* GABA<sub>B</sub>R; RNAi, RNA interference.

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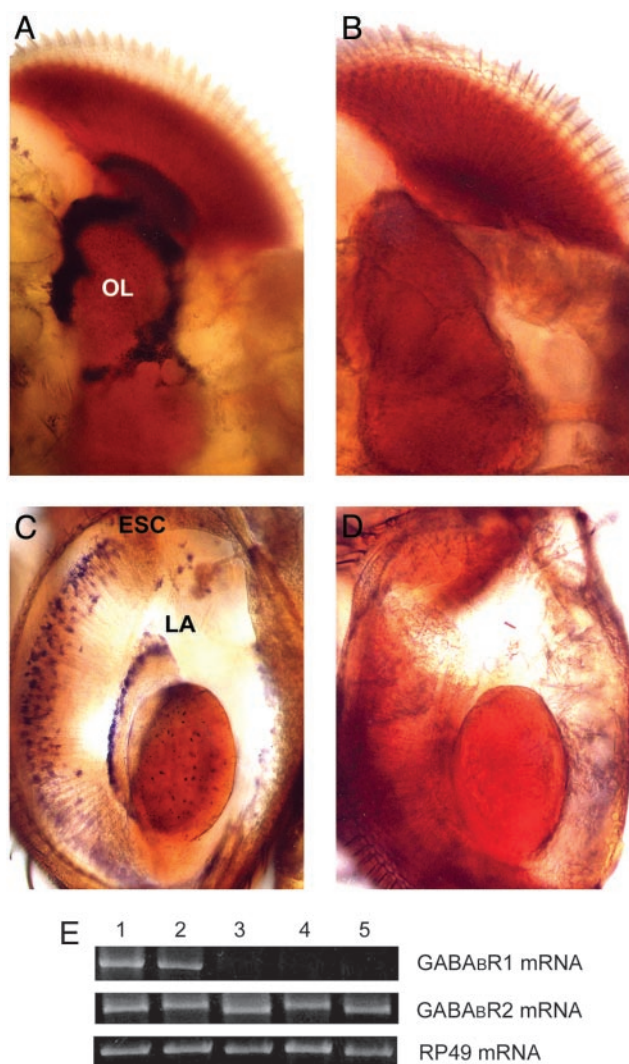
was 1%. All control flies were injected with the corresponding vehicle.

**Adult *Drosophila* RNAi.** In contrast to the cell-autonomous transgenic RNAi (26), the injectable RNAi appears to be effective throughout the cell body, i.e., cell-nonautonomous (10, 11), and is a preferable method for cell type-nonselective gene silencing. The preparation of dsRNA samples for injection proceeded as follows: an RT-PCR-amplified, 819-bp fragment of the *Drosophila* GABA<sub>B</sub>R1 gene (1401–2220) was used for preparation of “long” dsRNA. The PCR primers used were direct 5′-taatacagactactatatttgggtgtgcatgtgcacaa-3′ and reverse 5′-gtaatacagactactataggcatgacgtataacctcaa-3′. Experiments were also performed with “short” 21- to 22-bp dsRNA, which produced similar results (see *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org). Primers were designed with an attached T7 RNA polymerase promoter sequence. The specificity of the PCR product was verified with a restriction enzyme digestion (*Bgl*II site present at position 1811). This PCR product was directly used for *in vitro* transcription reaction with T7 RNA polymerase. RNA was heat-denatured at 80–85°C for 5 min and placed on ice. Gel electrophoresis was performed to assess the quality of dsRNA. The 750-bp GFP (Stratagene) dsRNA was used as a control (this gene is not normally expressed in *Drosophila*). Initially, two concentrations of “long” and “short” dsRNAs were used: 10 and 100 ng/μl. Because better results were obtained with a higher concentration, 100 ng/μl dsRNA was used in all subsequent experiments.

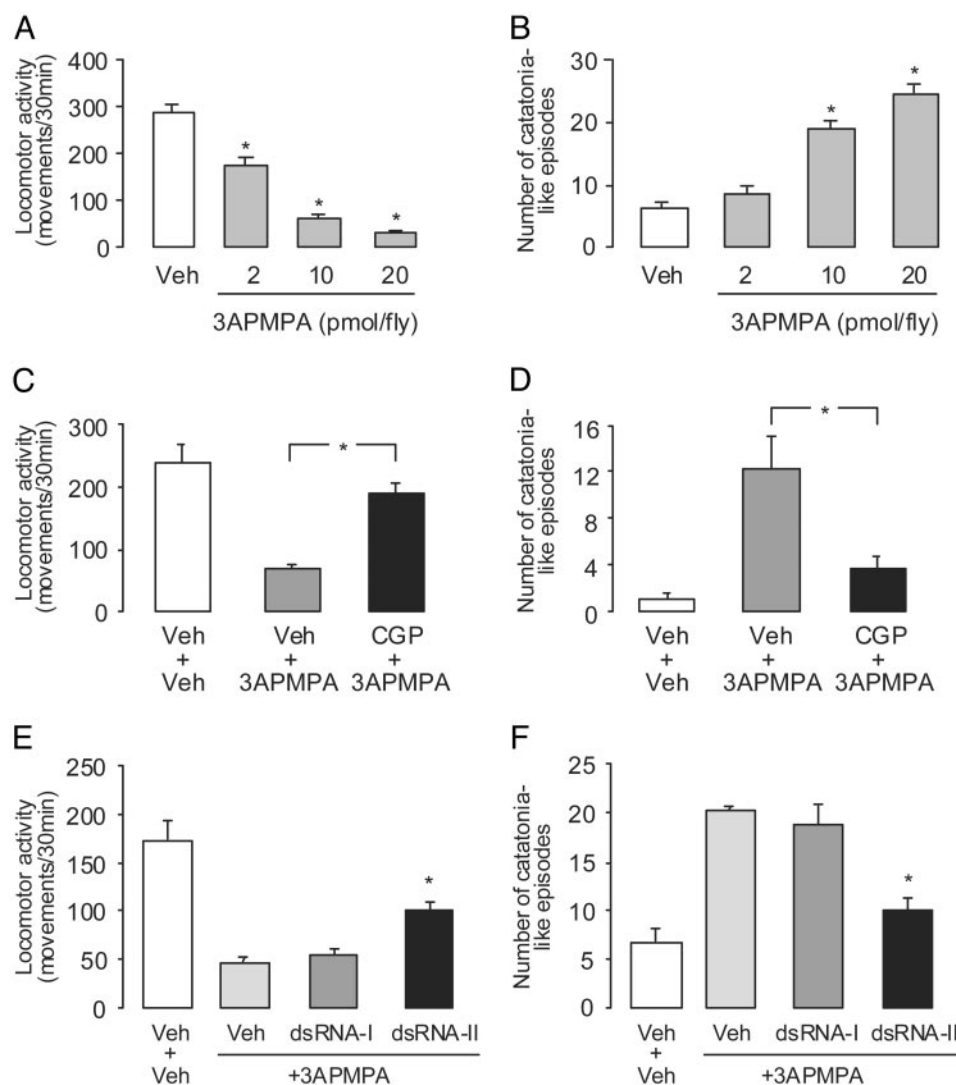
**Assay of Endogenous mRNAs.** For assaying the endogenous contents of mRNAs, we used the RT-PCR assay (10). The total RNA from a single fly was extracted with TRIzol reagent (Invitrogen). The specific primers used in PCR amplification were direct 5′-ttatttgggtgtgcatgtgcacaa-3′ and reverse 5′-gggatgacgtataacctcaa-3′ for GABA<sub>B</sub>R1, direct 5′-gcctgggaaactgacatggt-3′ and reverse 5′-ttgctccagttcgacaccca-3′ for GABA<sub>B</sub>R2, and direct 5′-atgacatcgccacgcataca-3′ and reverse 5′-tgtgtattccgaccaggttac-3′ for RP49 (internal control). For the *in situ* PCR, heads of flies were manually dissected on dry ice, placed in 500-μl PCR tubes with a fix buffer (12% formaldehyde in PBS), rinsed in PBS, treated with proteinase K in PBS (20 μg/ml) for 5 min at room temperature, fixed again for 5 min, and rinsed several times in PBS. Reverse transcription was performed in the presence of oligo(dT) with MMLV RT (United States Biochemical) in 50 μl of a total reaction mix. PCR DIG Labeling Mix (Roche Applied Science) was used in the PCR amplification reaction (90°C for 15 s, 57°C for 20 s, and 72°C for 30 s; 15 cycles total). The PCR primers used were direct 5′-ttatttgggtgtgcatgtgcacaa-3′ and reverse 5′-gggatgacgtataacctcaa-3′. Samples were washed in PBS three times (10 min per wash), incubated with anti-digoxigenin-AP Fab fragments (Roche Applied Science) at a dilution of 1:1,000 in PBS for 1 h at room temperature, and washed for 2 h in PBS (buffer changed four to five times). Color was developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche Applied Science). Samples were mounted in NaCl/glycerol solution.

**Behavior.** The *Drosophila* Activity Monitoring System (Trikinetics, Waltham, MA), coupled to a computer, was used to monitor the locomotor activity of individual flies. After administration of vehicle or drugs, flies remain immobile (because of CO<sub>2</sub> anesthesia and the actions of drugs) for a period. To quantify not only the total locomotor activity but also the duration of immobility, the system was slightly modified, i.e., the space in each individual recording tube was restricted to a length of 8 mm in the center of the photo beam. Flies were placed in the recording tubes

within 2 min of injection and the sampling time was set at 1-min intervals. After flies recovered from the injection, they gradually resumed locomotor activity. In preliminary studies, we established that up to 15 min of CO<sub>2</sub> anesthesia with or without injection does not result in any lasting effects on locomotor activity (e.g., >24 h). Nevertheless, different strains of flies express different degrees of basal locomotor activity; for Canton-S flies, we observed an average daytime activity >10 cpm (assayed over a period of several days). Thus, for this strain, we arbitrarily set the time of recovery from anesthesia as the first 1-min interval in which a fly produces 10 movements. Thereafter, the total locomotor activity was measured over the next 30-min period. The time to the first interval with more than 10 movements was used as the time of “awakening” and was analyzed



**Fig. 1.** GABA<sub>B</sub>R1 dsRNA destroys endogenous GABA<sub>B</sub>R1 mRNA. Three days before the GABA<sub>B</sub>R1 mRNA *in situ* PCR assay, the flies were injected with either GFP dsRNA (control; A and C) or GABA<sub>B</sub>R1 dsRNA (RNAi; B and D). GABA<sub>B</sub>R1 mRNA was found in A, the cortical layer of medulla and lobula neuropil of the optic lobe (OL; note the dark staining in A and its absence in B), and C [note the staining in the lamina outer ganglion cells (LA; the first neuropil of the optic lobe, which receives optic nerve fibers from compound eye photoreceptor cells) and the eye sensory cells (ESC)]. Note the absence of this staining in D. (E) RT-PCR assay of GABA<sub>B</sub>R1, GABA<sub>B</sub>R2, and RP49 (a ribosomal gene) mRNAs: lane 1, 24 h after vehicle; lane 2, 24 h after GFP dsRNA; lanes 3, 4, and 5, 24, 48, and 72 h after GABA<sub>B</sub>R1 dsRNA, respectively. Note the absence of GABA<sub>B</sub>R1 signals in lanes 3–5.



**Fig. 2.** Behavioral effects of the GABA<sub>B</sub> agonist 3-APMPA are attenuated by the GABA<sub>B</sub> antagonist CGP 54626 and by GABA<sub>B</sub>R1 dsRNA. The GABA<sub>B</sub> agonist 3-APMPA triggered a dose-dependent reduction of locomotor activity in flies (A) and increased the number of catatonia-like episodes (B). These actions of the GABA<sub>B</sub> agonist were reduced by pretreatment with CGP 54626 (0.2 nmol per fly) 1 h before 3-APMPA (10 pmol per fly) (C and D). Similar results were obtained in experiments with 2 pmol of 3-APMPA per fly (not shown). In RNAi experiments, 3 days before GABA<sub>B</sub> agonist injection, the flies were injected with vehicle or dsRNA [control GFP dsRNA (dsRNA-I) or GABA<sub>B</sub>R1 dsRNA (dsRNA-II)]. The behavioral effects of 10 pmol of 3-APMPA per fly were reduced by GABA<sub>B</sub>R1 dsRNA (E and F). The bars represent mean values  $\pm$  SEM. Significant differences (\*, Scheffé's test) between drug-treated flies and corresponding controls are: A and B,  $P \leq 0.001$  vs. vehicle (Veh;  $n = 12$  per group); C,  $P \leq 0.05$  ( $n = 6$ ); D,  $P \leq 0.001$  ( $n = 6$ ); and E and F,  $P \leq 0.01$  vs. other 3-APMPA-treated groups ( $n = 8$ ).

with respect to the drug treatment variable. In addition, the level of locomotor activity was recorded and quantified for consecutive periods. Typically, 6–10 flies per experimental group were used and experiments were repeated two to three times.

## Results

**Localization of GABA<sub>B</sub>R1 mRNA in Adult Flies and the Effect of dsRNA Injections.** Previous studies on GABA<sub>B</sub>R1 mRNA localization were performed in *Drosophila* embryos (15). Our studies localized GABA<sub>B</sub>R1 mRNA in the CNS of adult *Drosophila* (Fig. 1). In addition to the localization shown in Fig. 1, we observed a GABA<sub>B</sub>R1 mRNA signal in the giant fiber system, a pair of large bilaterally symmetrical interneurons that stimulate the visual jump–escape response and whose ventral and lateral dendrites receive mechanosensory and visual inputs (not shown). Injection of dsRNA complementary to endogenous GABA<sub>B</sub>R1 mRNA led to the complete destruction of endogenous GABA<sub>B</sub>R1 mRNA but not to the destruction of GABA<sub>B</sub>R2 mRNA; this

silencing of GABA<sub>B</sub>R1 was evident 24 h after dsRNA injection and persisted for at least 3 days (Fig. 1).

**Behavioral GABA<sub>B</sub> Pharmacology in Flies.** To evaluate the *in vivo* functioning of *Drosophila* GABA<sub>B</sub> receptors, we administered 3-APMPA (a GABA<sub>B</sub> agonist) and CGP 54626 (a GABA<sub>B</sub> antagonist) via intraabdominal injections and quantified the locomotor activity of injected flies. We observed that the injection of 3-APMPA into flies produces remarkable behavioral effects (see Movies 1–3, which are published as supporting information on the PNAS web site). After flies recovered from the injection (arbitrarily set in our system for monitoring the locomotor activity as the first 1-min interval in which a fly produces 10 movements; this time did not differ between vehicle- and drug-injected flies), 3-APMPA decreased locomotor activity measured over the subsequent 30-min period (Fig. 2). This GABA<sub>B</sub> agonist also caused intermittent jumps and periods of catatonia-like immobility that we also quantified. The behavioral



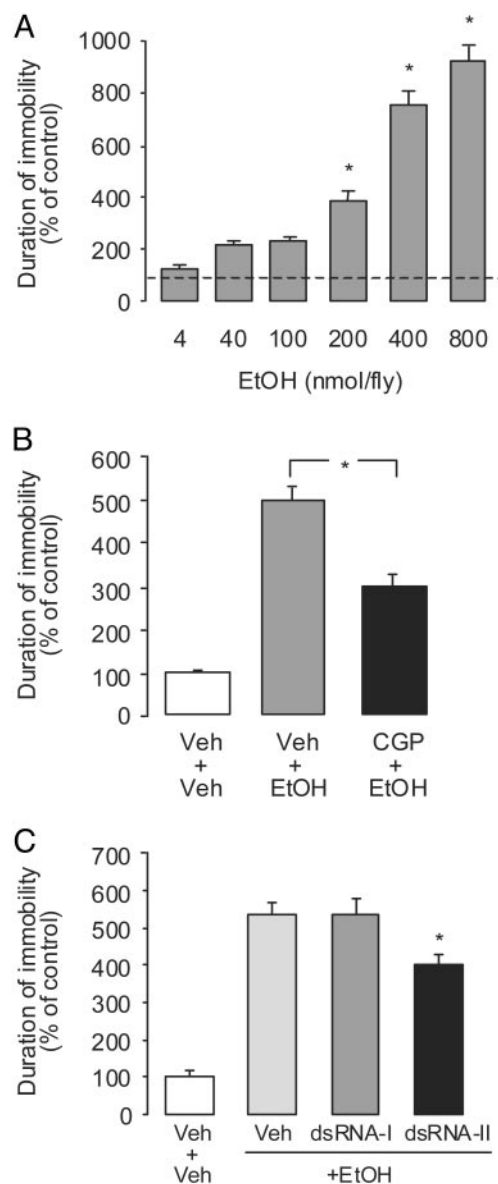
effects of 3-APMPA, i.e., decreased locomotor activity and increased catatonic immobility, were prevented by injecting the flies 1 h before with the GABA<sub>B</sub> antagonist CGP 54626 (Fig. 2), suggesting that, indeed, the behavioral effects of 3-APMPA are mediated by GABA<sub>B</sub> receptors.

**GABA<sub>B</sub>R1 RNAi Interferes with the Behavioral Actions of 3-APMPA.** Silencing of GABA<sub>B</sub>R1 by RNAi in adult flies did not produce any marked effects on spontaneous locomotor behavior and recovery from anesthesia (data not shown). Also, the administration of GABA<sub>B</sub> antagonist CGP 54626 to adult flies did not significantly affect their locomotion. However, behavioral response to 3-APMPA (a GABA<sub>B</sub> agonist) was significantly reduced in GABA<sub>B</sub>R1 dsRNA-injected flies (Fig. 2 and Fig. 5, which is published as supporting information on the PNAS web site). Our findings that endogenous GABA<sub>B</sub>R1 mRNA was completely destroyed by RNAi (Fig. 1), whereas the behavioral actions produced by the GABA<sub>B</sub> agonist were reduced but not completely abolished (Fig. 2), suggest that the half-life of this receptor protein might be significantly longer than the half-life of the mRNA. Because the currently available antibodies against the GABA<sub>B</sub>R1 subunit do not recognize *Drosophila* GABA<sub>B</sub>R1, we cannot at this time examine the possibility that the protein may have a longer half-life than the mRNA.

**Behavioral Effects of Ethanol Injections.** In our experimental model, ethanol was administered via injection and it caused a dose-dependent prolongation of immobility (e.g., anesthesia-like state; see Movies 1–3). The time from ethanol administration to the first interval with >10 movements was taken as the time of “awakening” (Fig. 3). The maximal effect of ethanol on awakening was observed with a dose of 0.8  $\mu$ mol per fly. Assayed 24 h after injection, higher concentrations resulted in significant toxicity (assayed as the number of dead flies 24 h after injection;  $\approx$ 20% with 1  $\mu$ mol of ethanol per fly and 100% with 1.6 of  $\mu$ mol per fly; data not shown).

**Behavior-Impairing Effects of Ethanol in *Drosophila* Involve GABA<sub>B</sub> Receptors.** Ethanol immobility and 3-APMPA-induced immobility, determined by our computer-assisted monitoring of locomotion, appear to be caused by different factors. Thus, ethanol-injected flies lie down and are nonresponsive to external stimuli (except for occasional leg movements) until they recover, stand, and gradually start to move. On the other hand, 3-APMPA-injected flies recover from injection under the CO<sub>2</sub> anesthesia almost as quickly as vehicle-injected flies; they stand up but do not move except for occasional jumps. Nevertheless, the behavior-impairing effect of ethanol was diminished by pretreatment with the GABA<sub>B</sub> antagonist CGP 54626 (Fig. 3). Surprisingly, this is similar to previous findings in mice; ethanol-induced motor impairment was investigated in mice by using their rotarod performance as the test response. Direct cerebellar microinfusion of a GABA<sub>B</sub> agonist and an antagonist produced dose-dependent accentuation and attenuation, respectively, of ethanol-induced acute motor impairment (16). Motor impairment in flies triggered by ethanol was also reduced by GABA<sub>B</sub>R1 RNAi (Fig. 3). Thus, our data suggest that the behavioral actions of 3-APMPA, and also in part ethanol, are mediated by an activation of GABA<sub>B</sub> receptors.

**In *Drosophila*, Rapid Tolerance to Ethanol Involves GABA<sub>B</sub> Receptors.** Recent studies in mice revealed that rapid tolerance to ethanol (measured as motor impairment on a rotarod apparatus) is also controlled by the GABA<sub>B</sub> receptors, but in a manner opposite from acute motor impairment. Thus, baclofen (a GABA<sub>B</sub> agonist) significantly blocked rapid tolerance to ethanol, and the blockade of rapid tolerance by baclofen was antagonized by previous administration of CGP 36742 or CGP 56433, which are

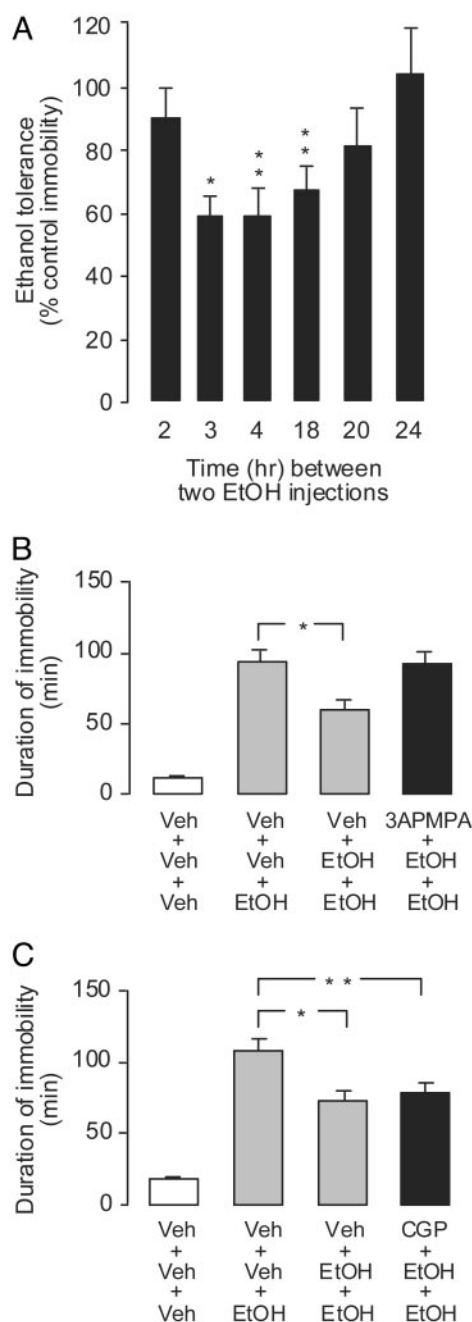


**Fig. 3.** CGP 54626 and GABA<sub>B</sub>R1 RNAi attenuate motor impairment induced by ethanol. (A) Injection of ethanol (EtOH) caused a dose-dependent prolongation of immobility (\*,  $P \leq 0.001$  vs. control, which is shown as 100%; Scheffé's test;  $n = 12$  per group). (B) CGP 54626 (0.2 nmol per fly) 1 h before EtOH (400 nmol per fly) shortened EtOH immobility (\*,  $P \leq 0.001$ ;  $n = 18$ –19). (C) dsRNA-II (as in Fig. 2), but not dsRNA-I, reduced motor impairment by EtOH (200 nmol per fly; \*,  $P \leq 0.05$  vs. other EtOH-treated groups;  $n = 14$ –24). Results are expressed as a percentage of corresponding control immobility (11–15 min), and bars represent mean values  $\pm$  SEM.

GABA<sub>B</sub> antagonists (17). We conducted similar experiments with *Drosophila*. In these studies, motor impairment induced by ethanol injection was reduced by previous ethanol administration; this tolerance persisted for  $\approx$ 18 h (Fig. 4). Similar to previous findings in mice, pretreatment with a GABA<sub>B</sub> agonist (i.e., 3-APMPA) blocked tolerance to ethanol in *Drosophila*, whereas CGP 54626 pretreatment did not significantly affect ethanol tolerance (Fig. 4).

## Discussion

Previous work by others has established *Drosophila* as a useful model organism for studies of ethanol (20), including the mechanisms of ethanol tolerance (23). Exposure to ethanol



**Fig. 4.** Repeated injections of ethanol led to ethanol tolerance that can be inhibited by pretreatment with 3-APMPA but not with CGP 54626. (A) Ethanol (EtOH; 800 nmol per fly) was injected twice (interval of 1–24 h), or flies were injected first with vehicle (Veh) and then with EtOH (nontolerant controls). The duration of immobility of flies treated with two EtOH is expressed as the percentage of immobility of the corresponding Veh + EtOH-treated controls (\*,  $P \leq 0.001$ ; \*\*,  $P \leq 0.01$ ; Student's *t* test;  $n = 7$ –16 flies per group and per time interval). The interval between the first injection (3-APMPA, 2 pmol per fly, or CGP 54626, 0.2 nmol per fly) and second injection (EtOH, 400 nmol per fly) (B and C) was 1 h, and the interval between the second and third injections was 4 h (to induce EtOH tolerance). All groups differed significantly from vehicle-only-injected flies; significant differences between EtOH-treated groups are indicated (\*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; Scheffé's test;  $n = 13$ ).

vapors was used to trigger behavioral alterations in flies (20), whereas genetic mutations or feeding flies on drugs mixed with the food was used to investigate specific molecular mechanisms. These studies point to a pivotal role for cAMP and cAMP-

related neurotransmitter systems in regulating the sensitivity of *Drosophila* to ethanol (21, 22). Our results clearly show an important role for the *Drosophila* GABA system, including the metabotropic cAMP-linked GABA<sub>B</sub> receptors, in the behavioral actions induced by ethanol treatment and indicate that *Drosophila* can be used to further characterize the mechanisms of the interactions between ethanol and GABA<sub>B</sub> receptors.

The inhibitory neurotransmitter GABA exerts its actions via two distinct receptor families: ionotropic receptors (GABA<sub>A</sub> and GABA<sub>C</sub>), which have been extensively studied, and metabotropic receptors (GABA<sub>B</sub>), which are less well characterized. The focus of research on the interactions of ethanol with the GABA system has been on GABA<sub>A</sub> receptors (27). However, it also has been shown that inwardly rectifying potassium channels coupled to GABA<sub>B</sub> receptors are targets of alcohol action in CNS neurons (28) and that GABA<sub>B</sub> receptors may participate in long-lasting potentiation of GABAergic synapses after a single *in vivo* ethanol exposure (29).

Recently, it was observed that the GABA<sub>B</sub> receptor agonist baclofen reduces alcohol craving in alcohol-dependent patients (18). Moreover, baclofen blocks the development of rapid tolerance to ethanol in mice (17), whereas GABA<sub>B</sub> antagonists attenuate the acute behavior-impairing actions of ethanol (16) and produce more severe ethanol withdrawal syndrome symptoms (19). An earlier report also indicated that inhibition of GABA<sub>B</sub> receptors may antagonize acute behavioral effects of ethanol (30). Hence, the GABA<sub>B</sub> receptor antagonist phaclofen administered s.c. to mice reduced the ataxic and hypothermic effects of subsequent i.p. injections of ethanol; however, one may question the specificity of phaclofen for GABA<sub>B</sub> receptors. Our findings in *Drosophila* support the role of these receptors because we observed the attenuation of alcohol's effects not only with a different GABA<sub>B</sub> receptor antagonist (i.e., CGP 54626) but also with RNAi-mediated GABA<sub>B</sub> receptor silencing. Nevertheless, the rescue of the acute motor-impairing action of ethanol by GABA<sub>B</sub>R1 antagonism was only partial, suggesting that the acute actions of ethanol are not mediated solely by GABA<sub>B</sub> receptors.

Similar to previous findings in mice (17), our experiments with *Drosophila* show that pretreatment with a GABA<sub>B</sub> receptor agonist also prevents the rapid tolerance to ethanol. In mice, certain doses of GABA<sub>B</sub> receptor antagonists (e.g., CGP 36742 and CGP 56433) facilitated ethanol tolerance. However, both in mice (17) and in flies, the doses of GABA<sub>B</sub> antagonists that antagonize the acute motor-impairing effects of ethanol did not facilitate rapid tolerance to ethanol. It has been proposed that a certain level of motor impairment on first ethanol exposure is required for the development of rapid tolerance (17). Thus, it is possible that a dose of CGP 54626 could be found that is ineffective in attenuating acute motor-impairing ethanol effects in flies and could facilitate rapid tolerance to ethanol.

We did not observe significant alterations of *Drosophila* locomotor activity after GABA<sub>B</sub> receptor antagonism or following GABA<sub>B</sub> receptor silencing in adult flies (i.e., by RNAi). This is different from major behavioral disturbances observed in GABA<sub>B</sub> receptor "knockout" mice (24, 25). It is possible that the pathologic behaviors of GABA<sub>B</sub> receptor-deficient mice are due in part to the developmental CNS alterations triggered by the absence of these receptors.

In insects, injections of dsRNA either into the pupae (31) or into adult organisms (10, 11, 32) lead to a cell-nonautonomous RNAi, which is preferable for studies of genes whose cell- or tissue-specificity of expression is unknown. It has been reported that functional GABA<sub>B</sub> receptors are present on insect motoneurons (33), in the CNS of *Drosophila* embryos (15), and our studies of localization of GABA<sub>B</sub>R1 mRNA (Fig. 1) postulate the presence of these receptors in the neuropil of the optic lobe and also in the giant fiber system. Whether these or some other neurons are responsible for mediating the behavioral actions of

ethanol and GABA<sub>B</sub> ligands is not clear at present. Additional work is also necessary to elucidate the exact mechanisms operative in GABA<sub>B</sub> receptor-mediated attenuation of behavioral responses to alcohol. We suggest that *Drosophila* may prove to be a good experimental model for these studies. For example, gene silencing in adult flies can be combined with pharmacological and biochemical tools to further characterize the functional role of individual subunits of GABA<sub>B</sub> receptors; i.e.,

D-GABA<sub>B</sub>R1, D-GABA<sub>B</sub>R2, and D-GABA<sub>B</sub>R3. A better understanding of the molecular mechanisms linking GABA<sub>B</sub> receptors to the behavioral effects of ethanol may lead to novel concepts concerning the molecular biology of drug abuse.

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